ENZYME KINETICS

Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems

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CHAPTER THREE

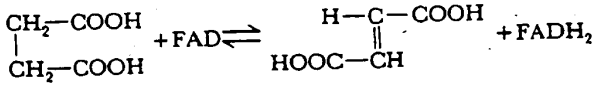
SIMPLE INHIBITION SYSTEMS

Any substance that reduces the velocity of an enzyme-catalyzed reaction can be considered to be an "inhibitor." The inhibition of enzyme activity is one of the major regulatory devices of living cells, and one of the most important diagnostic procedures of the enzymologist. Inhibition studies often tell us something about the specificity of an enzyme, the physical and chemical architecture of the active site, and the kinetic mechanism of the reaction. In our every day life, enzyme inhibitors can be found masquerading as drugs, antibiotics, preservatives, poisons, and toxins. In this chapter we examine three simple types of enzyme inhibitors. We assume that only a single substrate is involved in the reaction, and that only one type of inhibitor is present at any time. The effects of inhibitors on multisubstrate enzymes are discussed in Chapters Six and Nine. The effects of multiple inhibitors are discussed in Chapter Eight.

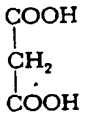
A. COMPETITIVE INHIBITION (SIMPLE INTERSECTING LINEAR COMPETITIVE INHIBITION)

A competitive inhibitor is a substance that combines with free enzyme in a manner that prevents substrate binding. That is, the inhibitor and the substrate are *mutually exclusive*, often because of true competition for the same site. A competitive inhibitor might be a nonmetabolizable analog or derivative of the true substrate, or an alternate substrate of the enzyme, or a product of the reaction.

Malonic acid is a classical example of a true competitive inhibitor. Malonic acid inhibits succinic dehydrogenase, which catalyzes the oxidation of succinic acid to fumaric acid, as shown below.



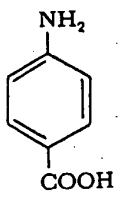
Malonic acid resembles succinic acid sufficiently to combine with the enzyme at the active site.



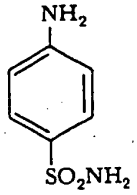
malonic acid

However, because malonic acid has only one methylene group, no oxidation-reduction can take place.

Another classical example of a competitive inhibitor is the sulfa drug sulfanilamide, which interferes with the biosynthesis of folic acid from the precursor *p*-amino-benzoic acid (PABA).



PABA



sulfanilamide

Figure III-1 illustrates several situations that lead to the mutually exclusive binding of S and I, Model 1 represents classical competitive inhibition. Models 2 to 4 yield the same results.

There are many examples of "competitive" inhibition by compounds that bear no structural relationship to the substrate. The inhibitor might be an end-product or near end-product of a metabolic pathway; the enzyme is one that catalyzes an early reaction (or a branch-point reaction) in the pathway. The phenomenon is called feedback inhibition. The inhibitor ("effector," "modulator," or "regulator") combines with the enzyme at a position other than the active (substrate) site. The combination of the inhibitor with the enzyme causes a change in the conformation (tertiary or quaternary structure) of the enzyme that distorts the substrate site and thereby prevents the substrate from binding (Model 5).

The inhibition of the hexokinase-catalyzed reaction between glucose and ATP by fructose or mannose is an example of competitive inhibition by alternate substrates. Glucose, fructose, and mannose are all substrates of hexokinase and can be converted to product (hexose-6-phosphate). All three hexoses combine with the enzyme at the same active site. Consequently, the

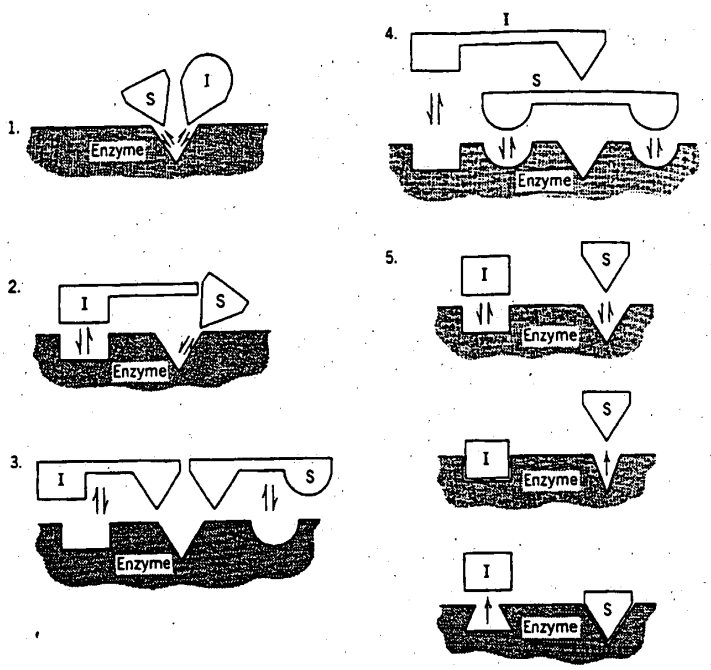


Fig. III-1. Models of competitive inhibition: S and I are mutually exclusive. (1) Classical model. S and I compete for the same binding site. I must resemble S structurally. (2) I and S are mutually exclusive because of steric hinderance. (3) I and S share a common binding group on the enzyme. (4) The binding sites for I and S are distinct, but overlapping. (5) The binding of I to a distinct inhibitor site causes a conformational change in the enzyme that distorts or masks the substrate binding site (and vice versa).

utilization of any one of the hexoses is inhibited in the presence of either of the other two.

The equilibria describing competitive inhibition are shown below. The competition and mutual exclusion of S and I are clearly seen.

$$\begin{array}{ccccc} E + S & \overset{K_s}{\rightleftharpoons} & ES & \overset{k_2}{\longrightarrow} & E + P \\ + & & & & \\ I & & & & \\ K \updownarrow & & & & \\ EI & & & & \end{array}$$

where $K_i = [E][I]/[EI]$, $K_S = [E][S]/[ES]$, and k_p = rate constant for the breakdown of ES to E + P.

The initial velocity of the reaction is proportional to the steady-state concentration of the enzyme-substrate complex, ES. All the enzyme species are reversibly connected. Consequently, we can predict that at any fixed unsaturating concentration of inhibitor (*a*) v_i (the velocity in the presence of a competitive inhibitor) can be made to equal v (the velocity in the absence of the inhibitor), but that a higher substrate concentration will be required (to obtain the same ES concentration), and (*b*) in the presence of an infinitely high (saturating) substrate concentration all the enzyme can be driven to the ES form. Consequently, the maximal initial velocity in the presence of the competitive inhibitor equals V_{max} (the maximum initial velocity in the absence of inhibitor). However, the apparent K_m (measured as [S] required for $\frac{1}{2} V_{max}$) will increase in the presence of a competitive inhibitor because at any inhibitor concentration, a portion of the enzyme exists in the EI form which has no affinity for S.

An expression relating v, V_{max}, [S], K_m, [I], and K_i in the presence of a competitive inhibitor can be derived from either rapid equilibrium or steady-state assumptions. This time we must recognize that the total enzyme $[E]_t$ is present in three forms: free enzyme, [E]; enzyme-substrate complex [ES]; and enzyme-inhibitor complex, [EI].

$$v = k_p[ES]$$

$$\frac{v}{[E]_t} = \frac{k_p[ES]}{[E] + [ES] + [EI]}$$

$$\frac{v}{k_p[E]_t} = \frac{\dfrac{[S]}{K_S}}{1 + \dfrac{[S]}{K_S} + \dfrac{[I]}{K_i}}$$

$$\boxed{\frac{v}{V_{max}} = \frac{\dfrac{[S]}{K_S}}{1 + \dfrac{[S]}{K_S} + \dfrac{[I]}{K_i}}} \qquad \text{(III-1)}$$

If we compare equation III-1 to the usual velocity equation II-8, we see that the denominator has gained an additional $[I]/K_i$ term representing the

EI complex. The numerator still has one term indicating that there is still only one product forming complex (ES). To obtain a more familiar form, the numerator and denominator of the right-hand part of equation III-1 can be multiplied by K_S and factored:

$$\frac{v}{V_{max}} = \frac{[S]}{K_S\left(1 + \frac{[I]}{K_i}\right) + [S]} \qquad \text{(III-2)}$$

We obtain the same final velocity equation for steady-state conditions; however, K_m replaces K_S. This is not surprising, since the steady-state assumption does not change the form of the velocity equation for the uninhibited reaction while the reaction between E and I to yield EI must be at equilibrium. (There is nowhere for EI to go but back to E+I).

The velocity equation differs from the usual Henri-Michaelis-Menten equation in that the K_m term is multiplied by the factor $(1+[I]/K_i)$. This confirms our original prediction that V_{max} is unaffected by a competitive inhibitor, but that the *apparent* K_m value is increased. The increase in the K_m value does *not* mean that the EI complex has a lower affinity for the substrate. The EI has no affinity at all for the substrate, while the affinity of E (the only form that can bind substrate) is unchanged. The apparent increase in K_m results from a distribution of available enzyme between the "full affinity" and "no affinity" forms. The factor $(1+[I]/K_i)$ may be considered as an [I]-dependent statistical factor describing the distribution of enzyme between the E and EI forms. There are systems in which EI has an altered affinity for S. This type of system, called partial competitive inhibition, is discussed in Chapter Four.

The effect of a competitive inhibitor on the kinetics of an enzyme-catalyzed reaction is illustrated in Figure III-2. The [I] was arbitrarily chosen as $3K_i$; $K_{m_{app}}$ then is $(1+3)K_m = 4K_m$. In the presence of the inhibitor it takes four times as much substrate to attain $0.5\,V_{max}$. In general:

$$\frac{[S]_i}{[S]} = \left(1 + \frac{[I]}{K_i}\right) \qquad \text{(III-3)}$$

where $[S]_i/[S]$ represents the ratio of substrate concentration required in the presence of inhibitor to substrate concentration required in the absence of inhibitor for any given velocity. A competitive inhibitor will increase $[S]_{0.9}$ and $[S]_{0.1}$. However, since both concentrations are increased by the same factor, the $[S]_{0.9}/[S]_{0.1}$ ratio is still 81 at all inhibitor concentrations. The

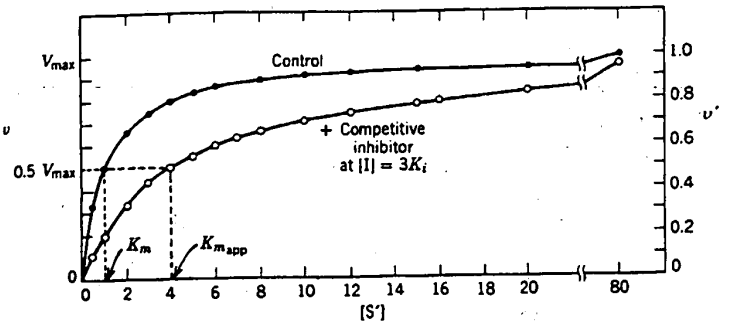


Fig. III-2. The v versus [S] plot in the presence and in the absence of a fixed concentration of a competitive inhibitor.

constant ratio is expected, since the form of the equation is unchanged; only the numerical value of K_m is changed.

An expression for the relative velocity or fractional activity in the presence and absence of a competitive inhibitor can be derived readily:

Let v_i = the initial velocity at a given [S] in the presence of inhibitor

v = the initial velocity at the same [S] in the absence of inhibitor

$\frac{v_i}{v} = a$ = the relative activity

$$\frac{v_i}{v} = a = \frac{\dfrac{V_{max}[S]}{K_m\left(1+\dfrac{[I]}{K_i}\right)+[S]}}{\dfrac{V_{max}[S]}{K_m+[S]}} \quad \text{or} \quad \boxed{a = \frac{K_m+[S]}{K_m\left(1+\dfrac{[I]}{K_i}\right)+[S]}} \qquad \text{(III-4)}$$

Relative velocity data are frequently expressed in terms of fractional inhibition (i) or "percent inhibition" ($i_{\%}$) where:

$$i = 1 - \frac{v_i}{v} = 1 - a \quad \text{and "percent inhibition"} \quad = 100i$$

$$\boxed{i = \frac{[I]}{[I]+K_i\left(1+\dfrac{[S]}{K_m}\right)}} \qquad \text{(III-5)}$$

Effect of Concentration Range on Degree of Inhibition

A point not always appreciated is that the degree of inhibition caused by an *n-fold excess* of competitive inhibitor is maximum when both [I] and [S] are very high compared to K_i and K_m, respectively. To put it another way, if [S] is very low compared to K_m, an excess of competitive inhibitor will not cause much inhibition even though K_i is of the same order of magnitude as K_m. A simple example illustrates the point. Suppose $[S] = 0.01K_m$ and $K_i = K_m$. What is the degree of inhibition caused by a tenfold excess of inhibitor (i.e., by an $[I] = 10[S]$)?

$$i = \frac{0.1K_m}{0.1K_m + K_m(1+0.01)} = \frac{0.1K_m}{1.11K_m} = 0.09$$

$$i_{\%} = 9\%$$

In other words, the inhibited velocity is 91% of the control velocity; we observe only 9% inhibition. On the other hand, when $[S] = 10K_m$, a tenfold excess of inhibitor ($[I] = 100K_m$) will inhibit 90% as shown below.

$$i = \frac{100K_m}{100K_m + K_m + 10K_m} = \frac{100K_m}{111K_m}$$

$$\therefore \quad i = 0.90 \quad \text{or} \quad i_{\%} = 90$$

Figure III-3 shows the effect of increasing competitive inhibitor concentration on the initial velocity at three different substrate concentrations. The degree of inhibition depends on the substrate concentration, decreasing as [S] increases, as predicted by equation III-5. To obtain 50% inhibition:

$$\boxed{[I]_{0.5} = \left(1 + \frac{[S]}{K_m}\right)K_i} \qquad \text{(III-6)}$$

Similarly, we can show that $[I]_{0.9}$ and $[I]_{0.1}$ (the inhibitor concentrations required for 90% and 10% inhibition, respectively) are:

$$[I]_{0.9} = 9\left(1 + \frac{[S]}{K_m}\right)K_i, \qquad [I]_{0.1} = \frac{1}{9}\left(1 + \frac{[S]}{K_m}\right)K_i$$

Thus the $[I]_{0.9}/[I]_{0.1}$ ratio is always 81, regardless of the substrate concentration or the values of K_m and K_i.

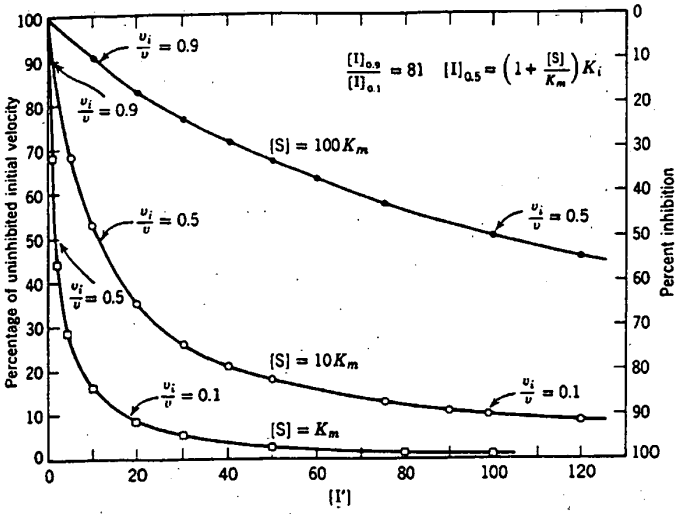


Fig. III-3. Relative activity as a function of competitive inhibitor concentration in the presence of different fixed concentrations of substrate.

Reciprocal Plot for Competitive Inhibition Systems

The velocity equation for competitive inhibition in reciprocal form is:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}} \qquad \text{(III-7)}$$

Thus the slope of the plot increases by the factor $(1+[I]/K_i)$ (which multiplied K_m in the original equation), but the $1/v$-axis intercept remains $1/V_{max}$. For each inhibitor concentration, a new reciprocal plot can be drawn. As [I] increases, the "plus inhibitor" curves increase in slope (Fig. III-4) pivoting counterclockwise about the point of intersection with the control curve (at $1/V_{max}$ on the $1/v$-axis). Because the initial velocity can be driven to zero by a saturating inhibitor concentration, the limiting plot will be a vertical line on the $1/v$ axis. As [I] increases, the intercept on the $1/[S]$ axis moves closer to the origin; that is, $K_{m_{app}}$ continually increases. The K_i can be calculated from the slope of any reciprocal plot or from any $K_{m_{app}}$. However, a replot as described below is better.

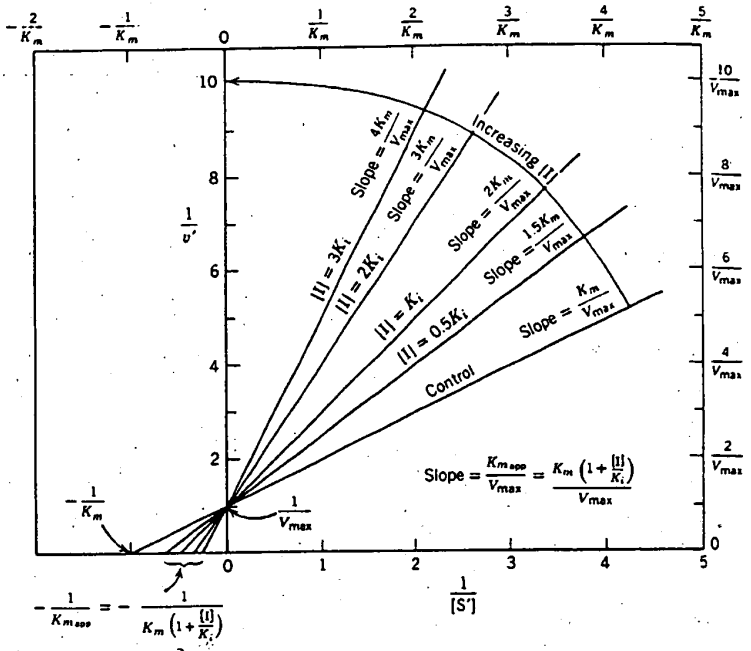


Fig. III-4. The $1/v$ versus $1/[S]$ plot in the presence of different fixed concentrations of a competitive inhibitor.

Replots of Slope and $K_{m_{app}}$ Versus [I]

The slope of the reciprocal plot in the presence of a competitive inhibitor is given by:

$$slope_{1/S} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) \quad \text{or} \quad \boxed{slope_{1/S} = \frac{K_m}{V_{max}K_i}[I] + \frac{K_m}{V_{max}}} \tag{III-8}$$

A replot of the slope of each reciprocal plot versus the corresponding inhibitor concentration will be a straight line with a slope of $K_m/V_{max}K_i$ and an intercept on the $slope_{1/S}$-axis of K_m/V_{max} (i.e., control slope at $[I]=0$) (Fig. III-5*a*). When $slope_{1/S}=0$, the intercept on the [I]-axis gives $-K_i$. For

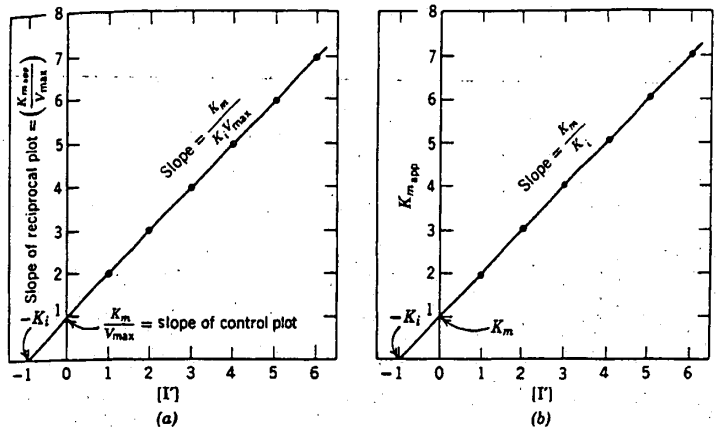


Fig. III-5. Replots of data taken from the reciprocal plot. (*a*) $slope_{1/S}$ versus [I]. (*b*) $K_{m_{app}}$ versus [I].

convenience the slope of the reciprocal plots can be read off directly as the ratio (absolute values) of the vertical axis intercept to horizontal axis intercept. A linear $slope_{1/S}$ versus [I] replot distinguishes pure competitive inhibition from partial competitive inhibition. The latter gives hyperbolic $slope_{1/S}$ versus [I] replots (Chapter Four). The $K_{m_{app}}$ is also a linear function of the inhibitor concentration in pure competitive systems:

$$K_{m_{app}} = \frac{K_m}{K_i}[\mathrm{I}] + K_m \qquad \text{(III-9)}$$

A replot of $K_{m_{app}}$ versus [I] has intercepts of K_m (on the $K_{m_{app}}$-axis) and $-K_i$ (on the [I]-axis) (Fig. III-5*b*).

Dixon Plot for Competitive Inhibition: $1/v$ Versus [I]

The Dixon plot is used frequently to identify the type of inhibition and to determine the K_i value. The velocity equation for competitive inhibition may be converted to a linear form in which the varied ligand is [I]. Starting

with the reciprocal equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}[S]}\left(1 + \frac{[I]}{K_i}\right) + \frac{1}{V_{max}} = \frac{K_m}{V_{max}[S]} + \frac{K_m[I]}{V_{max}[S]K_i} + \frac{1}{V_{max}}$$

$$\boxed{\frac{1}{v} = \frac{K_m}{V_{max}[S]K_i}[I] + \frac{1}{V_{max}}\left(1 + \frac{K_m}{[S]}\right)} \qquad \text{(III-10)}$$

A plot of $1/v$ versus [I] at some unsaturating [S] will yield a straight line with a positive slope as shown in Figure III-6*a*. If the inhibition is known to be competitive and V_{max} is known, a horizontal line at a height of $1/V_{max}$ can be drawn. The $-[I]$ value at the intersection of two lines gives K_i as shown below. When $1/v = 1/V_{max}$:

$$\frac{1}{V_{max}} = \frac{K_m[I]}{V_{max}[S]K_i} + \frac{1}{V_{max}}\left(1 + \frac{K_m}{[S]}\right)$$

$$1 = \frac{K_m[I]}{[S]K_i} + 1 + \frac{K_m}{[S]}$$

$$-\frac{K_m[I]}{[S]K_i} = \frac{K_m}{[S]} \qquad \text{or} \qquad [I] = -K_i$$

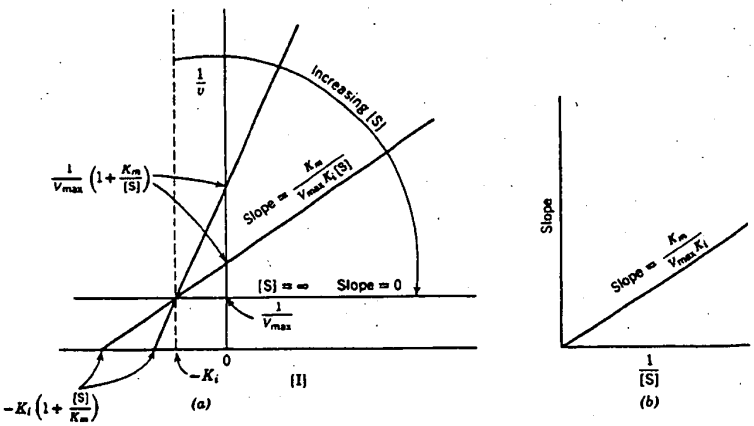


Fig. III-6. (*a*) Dixon plot for a competitive inhibitor: $1/v$ versus [I] in the presence of different fixed concentrations of substrate. If V_{max} is known, a horizontal line at a height of $1/V_{max}$ can be drawn directly. (*b*) Replot of the slopes of the Dixon plot.

The horizontal line at $1/V_{max}$ also signifies that at an infinitely high [S], increasing the inhibitor concentration will have no effect on v. If the inhibition is not known for sure to be competitive, or if V_{max} is unknown, another series of experiments at a different unsaturating [S] will yield a second line with a different positive slope. The intersection of the $[S]_1$ and $[S]_2$ lines, where $1/v_1 = 1/v_2$, gives K_i as shown below. When $1/v_1 = 1/v_2$:

$$\frac{K_m[I]}{V_{max}[S]_1 K_i} + \frac{[S]_1 + K_m}{V_{max}[S]_1} = \frac{K_m[I]}{V_{max}[S]_2 K_i} + \frac{[S]_2 + K_m}{V_{max}[S]_2} \quad \text{or} \quad \frac{[I] + K_i}{[S]_1} = \frac{[I] + K_i}{[S]_2}$$

The equation above holds true only if $[S]_1 = [S]_2$, which is not the case, or when both sides equal zero; that is, when $[I] = -K_i$.

The slope of the Dixon plot is given by:

$$slope = \frac{K_m}{V_{max}K_i}\frac{1}{[S]} \qquad \text{(III-11)}$$

Thus a replot of *slope* versus the corresponding $1/[S]$ (Fig. III-6*b*) will be a straight line through the origin with a slope of $K_m/V_{max}K_i$.

The family of Dixon plots for pure competitive inhibition intersects above the [I]-axis at $[I] = -K_i$ and $1/v = 1/V_{max}$. Certain types of mixed inhibition systems also yield lines that intersect above the [I]-axis. Consequently, a Dixon plot such as that shown in Figure III-6*a* establishes only that the inhibition is neither noncompetitive nor uncompetitive. Other plots for competitive inhibition systems are described in Chapter Four.

General Principles

A competitive inhibitor acts only to increase the apparent K_m for the substrate. As [I] *increases, $K_{m_{app}}$ increases. The V_{max} remains unchanged, but in the presence of a competitive inhibitor a much greater substrate concentration is required to attain any given fraction of V_{max}. The v_i may be considered equal to V_{max} when* $[S] \geq 100K_{m_{app}}$.

The degree of inhibition caused by a competitive inhibitor depends on [S], [I], K_m, *and K_i. An increase in* [S] *at constant* [I] *decreases the degree of inhibition. An increase in* [I] *at constant* [S] *increases the degree of inhibition. The lower the value of K_i, the greater is the degree of inhibition at any given* [S] *and* [I]. *The K_i is equivalent to the concentration of* I *that doubles the slope of the $1/v$ versus $1/[S]$ plot. (K_i is not equivalent to the* [I] *that yields* 50% *inhibition*).

Integrated Rate Equation in the Presence of a Competitive Inhibitor

If the reaction has a very large K_{eq} and none of the products have an appreciable affinity for the enzyme, then the integrated Henri-Michaelis-Menten equation in the presence of a competitive inhibitor can be written as:

$$\frac{2.3}{t}\log\frac{[S]_0}{[S]} = -\frac{1}{K_m\left(1+\frac{[I]}{K_i}\right)}\frac{[P]}{t} + \frac{V_{max}}{K_m\left(1+\frac{[I]}{K_i}\right)} \qquad \text{(III-12)}$$

where $[P] = [S]_0 - [S]$. The equation assumes that [I] remains constant as [S] decreases. Consequently, I cannot be an alternate substrate. The determination of [P] at various times during the course of the reaction will permit $K_{m_{app}}$ and V_{max} to be determined. A family of curves can be obtained for different inhibitor concentrations (Fig. III-7). The values of K_m and K_i can be determined from appropriate replots of the slopes or vertical axis intercepts.

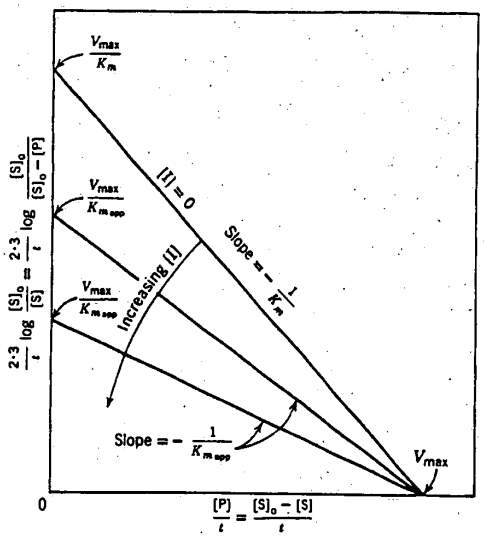


Fig. III-7. Plot of the integrated velocity equation in the presence of a competitive inhibitor.

Competitive Inhibition and Total Velocity with Mixed Alternate Substrates

When a single enzyme acts on two different substrates, and both are present simultaneously, each will act as a competitive inhibitor with respect to the other. If the products of the two substrates can be distinguished from each other, the system may be treated by the usual competitive inhibition relationships. If, on the other hand, the products are identical, or appear so by the assay method used, then the situation becomes more complex. For example, suppose the enzyme is a nonspecific phosphatase that catalyzes the reactions A→Q+P and B→R+P where A and B are two phosphate esters, Q and R are two distinct alcohols, and P is inorganic phosphate. In the presence of A and B the equilibria are:

$$\begin{array}{ccccccccc} & & & & \overset{K_A}{\rightleftharpoons} & & \overset{k_{p_A}}{\longrightarrow} & & \\ E & + & A & & EA & & E & + Q + & P \\ + & & & & & & & & \\ B & & & & & & & & \\ \Big\uparrow\Big\downarrow K_B & & & & & & & & \\ EB & & & & \overset{k_{p_B}}{\longrightarrow} & & E & + R + & P \end{array}$$

If the rate of P formation is measured, the observed velocity, v_i, is the sum of two reactions:

$$v_i = k_{p_A}[EA] + k_{p_B}[EB] \qquad \text{and} \qquad \frac{v_i}{[E]_t} = \frac{k_{p_A}[EA] + k_{p_B}[EB]}{[E] + [EA] + [EB]}$$

Assuming rapid equilibrium conditions (where $K_A = K_{m_A}$ and $K_B = K_{m_B}$):

$$v_i = \frac{V_{max_A}\dfrac{[A]}{K_{m_A}} + V_{max_B}\dfrac{[B]}{K_{m_B}}}{1 + \dfrac{[A]}{K_{m_A}} + \dfrac{[B]}{K_{m_B}}} \qquad \text{(III-13)}$$

where $V_{max_A} = k_{p_A}[E]_t$ = the maximal velocity with A as a substrate
$V_{max_B} = k_{p_B}[E]_t$ = the maximal velocity with B as the substrate

Equation III-13 can be rearranged to:

$$v_i = \frac{V_{max_A}[A]}{K_{m_A}\left(1+\frac{[B]}{K_{m_B}}\right)+[A]} + \frac{V_{max_B}[B]}{K_{m_B}\left(1+\frac{[A]}{K_{m_A}}\right)+[B]} \qquad \text{(III-14)}$$

Thus one enzyme that accepts two substrates will yield results identical to two enzymes, each of which is specific for one substrate but is competitively inhibited by the other's substrate.

The v_i can be expressed in terms of v_A (the velocity observed at the given [A] in the absence of B) and v_B (the velocity observed at the given [B] in the absence of A).

Substituting:

$$V_{max_A} = v_A\left(1+\frac{K_{m_A}}{[A]}\right) \quad \text{and} \quad V_{max_B} = v_B\left(1+\frac{K_{m_B}}{[B]}\right)$$

into equation III-13 and simplifying:

$$v_i = \frac{v_A\left(1+\frac{[A]}{K_{m_A}}\right)+v_B\left(1+\frac{[B]}{K_{m_B}}\right)}{1+\frac{[A]}{K_{m_A}}+\frac{[B]}{K_{m_B}}} \qquad \text{(III-15)}$$

If A and B are equally acceptable substrates (i.e., same K_m and V_{max}), then the v_i observed in the presence of any given mixture of A and B will be the same as the v observed with either A or B alone at the same total specific concentration. For example, if $V_{max_A}=1$ and $V_{max_B}=1$, then at $[A]=0.2K_{m_A}$ plus $[B]=0.6K_{m_B}=0.6K_{m_A}$, v_i equals 0.444. This is the same v observed at $[A]=0.8K_{m_A}$ and $[B]=0$, or $[B]=0.8K_{m_B}$ and $[A]=0$. On the other hand, v_i is *always less than* the sum of the velocities observed with each substrate alone at a given concentration. In the example above, $v_A=0.167$ at $[A]=0.2K_{m_A}$ and $[B]=0$. The $v_B=0.375$ at $[B]=0.6K_{m_B}$ and $[A]=0$. The sum, $v_A+v_B=0.542$, is greater than the observed v_i of 0.444 at $[A]=0.2K_{m_A}$ plus $[B]=0.6K_{m_B}$. The fact that v_i is always less than v_A+v_B may seem odd at first. But suppose A and B are really the same compound and $V_{max}=1$. At $[A]=K_{m_A}$, $v_A=0.5$. At $[B]=K_{m_B}$, $v_B=0.5$. $v_A+v_B=1$, yet we know that at $[A]=2K_{m_A}$ or $[B]=2K_{m_B}$, v is only 0.667. Note that $v_i<v_A+v_B$ holds regardless of the relative values of K_{m_A}, K_{m_B}, V_{max_A} and V_{max_B}. As with ordinary competitive inhibition, the degree of inhibition (in this case, the difference between v_i and v_A+v_B)

decreases as the concentration of either A or B becomes small compared to the respective K_m values. The maximum difference between v_i and $v_A + v_B$ is observed when both A and B are present at concentrations that are very high compared to their respective K_m values.

If the specific concentrations of A and B are equal:

$$v_i = \frac{v_A\left(1+\frac{[A]}{K_{m_A}}\right)+v_B\left(1+\frac{[A]}{K_{m_A}}\right)}{1+\frac{[A]}{K_{m_A}}+\frac{[A]}{K_{m_A}}} = (v_A+v_B)\frac{1+\frac{[A]}{K_{m_A}}}{1+\frac{2[A]}{K_{m_A}}} \qquad \text{(III-16)}$$

Thus when $[A]/K_{m_A}$ and $[B]/K_{m_B}$ are very small, $v_i \cong v_A + v_B$. As $[A]/K_{m_A}$ and $[B]/K_{m_B}$ increase (but remain equal), v_i increases to a limit of $(v_A+v_B)/2$.

An interesting relationship can be derived for the special case where A and B are present at equimolar concentrations (not equal specific concentrations). If $[A]=[B]$:

$$v_i = \frac{V_{max_A}\frac{[A]}{K_{m_A}} + V_{max_B}\frac{[A]}{K_{m_B}}}{1+\frac{[A]}{K_{m_A}}+\frac{[A]}{K_{m_B}}}$$

Dividing numerator and denominator by [A]:

$$v_i = \frac{\frac{V_{max_A}}{K_{m_A}} + \frac{V_{max_B}}{K_{m_B}}}{\frac{1}{[A]}+\frac{1}{K_{m_A}}+\frac{1}{K_{m_B}}}$$

When [A] and [B] are very high compared to their respective K_m values, the $1/[A]$ term becomes negligible and the observed combined velocity is maximal.

$$v_i \cong \frac{\frac{V_{max_A}}{K_{m_A}} + \frac{V_{max_B}}{K_{m_B}}}{\frac{1}{K_{m_A}}+\frac{1}{K_{m_B}}} = V_{max_i}$$

or

$$\boxed{\frac{K_{m_B}}{K_{m_A}} = \frac{V_{max_B} - V_{max_i}}{V_{max_i} - V_{max_A}}} \qquad \text{(III-17)}$$

Thus the relative K_m values can be determined from three measurements, namely, V_{max_A}, V_{max_B}, and V_{max_t} (maximal mixed velocity with an equimolar mixture of A and B).

If two specific enzymes are present and each is unaffected by the other's substrate, then v_t will equal $v_A + v_B$. However, there are at least five conditions where a mixture of two enzymes yields $v_t < v_A + v_B$: (*a*) each enzyme is catalytically active with only one of the substrates, but is competitively inhibited by the other substrate (equation III-14); (*b*) each enzyme is catalytically active with only one of the substrates but one of the enzymes (e.g., the A-specific enzyme) is competitively inhibited by the other's substrate (B); (*c*) one enzyme is catalytically active with only one of the substrates (e.g., A) and is unaffected by the other substrate (B), and the second enzyme is nonspecific; (*d*) one enzyme is catalytically active with only one of the substrates (e.g., A), but is competitively inhibited by the other substrate, and the second enzyme is nonspecific; (*e*) two nonspecific enzymes. Under conditions *c* and *d*, nonlinear reciprocal and Eadie-Scatchard plots may be seen when the varied substrate is the one acted on by both enzymes. Under condition *e*, nonlinear plots may be seen for both substrates. Conditions *a* and *b* yield linear plots for both substrates. (In both cases, there is only one enzyme active on a given substrate.) Additional distinctions may be made if v is measured as the rate of unique product (Q and R) formation. Under conditions *a*, *d*, and *e*, a saturating concentration of either substrate will inhibit completely the formation of the unique product of the other substrate. Under condition *b*, a saturating concentration of one substrate (e.g., B) will inhibit completely the formation of the unique product of the other substrate (e.g., Q from A), but not vice versa (i.e., [A] will have no effect on R formation from B). Under condition *c*, a saturating concentration of one substrate (e.g., A) will inhibit completely the formation of the unique product of the other substrate (e.g., R from B). The reverse experiment yields partial inhibition. A saturating concentration of B will inhibit only the activity of the nonspecific enzyme on A. The activity of the A-specific enzyme is unaffected.

The mixed substrate phenomenon was applied in an interesting way to a study of NH_4^+ transport by *Penicillium chrysogenum*. In this study methylammonium-^{14}C was used as an NH_4^+ analog. The K_m for methylammonium-^{14}C transport was 10^{-5} M, and the V_{max} was 10 μmoles$\times$g dry weight cells$^{-1}\times$min^{-1}. The physiologically important substrate of the transport system, NH_4^+ was a potent inhibitor of methylammonium-^{14}C transport ($K_{i_{NH_4^+}} = 2.5\times10^{-7}$ M). The K_i value for NH_4^+ as an inhibitor was assumed to be equivalent to the K_m value for NH_4^+ as a substrate. The mixed substrate method was used to estimate V_{max} for NH_4^+ transport. Varying concentrations of NH_4^+ (10^{-5} to 10^{-4} M) were mixed with a constant con-

centration(10^{-4} *M*) of methylammonium-^{14}C and the uptake of the methyl ammonium-^{14}C from each mixture was determined over a period of time. The results are shown in Figure III-8. During the early stages of the assay, NH_4^+ almost completely displaced methylammonium-^{14}C from the transport system. During the same time period, NH_4^+ was transported into the cells thereby reducing its external concentration. This resulted in a progressive decrease in the inhibition of methylammonium-^{14}C transport with time. When the NH_4^+ had been depleted, the methylammonium-^{14}C transport rate attained the control rate. The lag period (between zero-time and the time when the methylammonium-^{14}C transport rate attained the control rate) was taken as the time required to transport the NH_4^+ present. The length of the lag period was proportional to the initial NH_4^+ concentration.

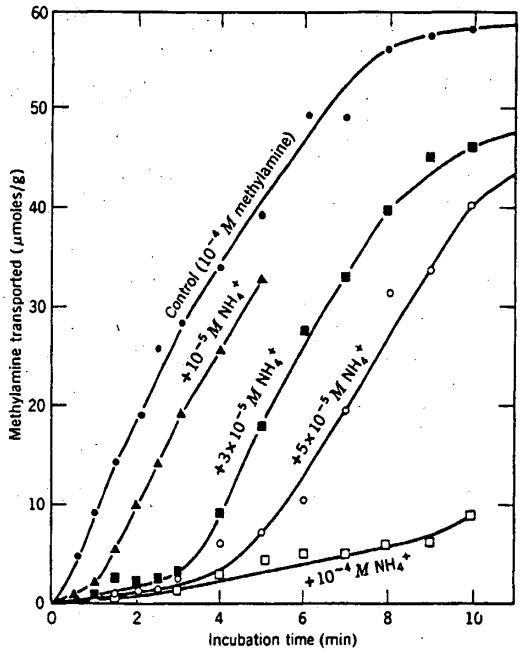


Fig. III-8. Effect of NH_4^+ on methylamine-^{14}C transport by *Penicillium chrysogenum*. Methylamine-^{14}C is excluded from the transport system as long as NH_4^+ is present in the medium. [Redrawn from Hackette, S. L., Skye, G. E., Burton, C. and Segel, I. H., *J. Biol. Chem.* **245**, 4241, (1970).] (See Dixon, M. and Webb, E. C., *Enzymes*, 2nd ed., Ch. 4, p. 88 for another example observed by Willstätter *et al.*, in 1927.)

The rate thus calculated was estimated as V_{max} for NH_4^+ transport. The estimate assumes that (*a*) 10^{-4} *M* methylammonium had little effect on the rate of NH_4^+ transport from solutions containing 10^{-5} to 10^{-4} *M* NH_4^+, and (*b*) the rate of NH_4^+ transport was essentially constant over the lag period. Assumption *a* is valid because the affinity of the transport system for NH_4^+ is 40-fold greater than the affinity for methylammonium. Assumption *b* is also valid because more than 97% of the NH_4^+ would have been transported before its concentration decreased to the K_m value.

Apparent Competitive Inhibition by Carrier Dilution (Isotope Competition)

In assays employing radioactive substrates, the addition of unlabeled substrate will produce the same *apparent* degree of inhibition as an equivalent amount of an alternate substrate with the same K_m value, or a nonsubstrate competitive inhibitor whose K_i value equals the K_m of the radioactive substrate. This method can be used to obtain a rapid comparison of the relative affinities of a variety of alternate substrates or nonsubstrate inhibitors. The carrier dilution method is illustrated below.

Suppose an enzyme catalyzes a reaction with a certain substrate, S, where $K_m = 2\times10^{-5}$ *M* and $V_{max} = 25$ μmoles/min. If radioactive S is used with a specific activity of 300,000 cpm/μmole, then at $[S] = K_m$, for example, the initial velocity will be $0.5\,V_{max}$, or 12.5 μmoles/min. The experimental raw data value of v would be (12.5 μmoles/min)×(3×10^5 cpm/μmole) = 37.5×10^5 cpm/min. If a tenfold excess of unlabeled substrate (2×10^{-4} *M*) is added to the assay mixture together with the radioactive substrate, the specific activity of the substrate will be reduced to 1/11 of the original specific activity. The new specific activity will be 27,272 cpm/μmole. The new [S] will be $11K_m$ and the new velocity will be $11/12\,V_{max}$, or 22.9 μmoles/min. The raw data value of v will be (22.9 μmoles/min)×(27,272 cpm/μmole) = 6.25×10^5 cpm/min. Compared to the original rate of 37.5×10^5 cpm/min, we observe an *apparent* 83.3% inhibition.

$$a = \frac{6.25\times10^5}{37.5\times10^5} = 0.167 \qquad i = 1 - 0.167 = 0.833$$

This corresponds to an *apparent* v of 2.08 μmoles/min. The true velocity, of course, has not decreased. It has increased on adding the additional substrate. However, v will appear to decrease if the raw data rate in terms of cpm/min are compared to the original raw data rate, or if the rate in terms of μmoles/min is calculated using the original, undiluted specific activity.

Now, let us calculate the degree of inhibition caused either by a tenfold

excess of an alternate substrate where the K_m value equals the K_m of the radioactive substrate, or by a tenfold excess of a nonsubstrate competitive inhibitor where K_i equals the K_m of the substrate.

$$v = \frac{(25)(K_m)}{K_m\left(1+\frac{10K_i}{K_i}\right)+K_m} = \frac{25K_m}{12K_m} = 2.08\ \mu\text{moles/min}$$

The specific activity of the substrate is unchanged, so the observed raw data value of v would be $(2.08\ \mu\text{moles/min}) \times (3\times10^5\ \text{cpm}/\mu\text{mole}) = 6.25\times10^5$ cpm/min. Thus whether we compare the raw data velocities in terms of cpm/min, or velocities in terms of μmoles/min, the inhibitor and the unlabeled substrate produced the same degree of inhibition—real in the presence of the inhibitor, but only apparent in the presence of the excess unlabeled substrate. If the inhibitor produced a lower degree of inhibition than an equivalent amount of excess substrate, then we could conclude that $K_i > K_m$. If the inhibitor produced a greater degree of inhibition, then K_i must be less than K_m. It is not necessary that K_m and V_{max} be known to compare affinities by this method. However, the maximum degree of inhibition with either unlabeled substrate or inhibitor will be observed when the concentration of radioactive substrate is high compared to K_m.

Isotope competition can be used to determine unknown concentrations of unlabeled substrate in solutions known to be free of real inhibitors. If we substitute $[S]/K_m$ for $[I]/K_i$ in equation III-4 we obtain:

$$\boxed{\frac{v_i}{v} = a = \frac{K_m + [S^*]}{K_m + [S] + [S^*]}} \qquad \text{(III-17a)}$$

where $[S^*]$ = the known concentration of radioactive substrate in the assay mixture

$[S]$ = the added unknown concentration of unlabeled substrate

$v_i/v = a$ = the relative activity in terms of cpm/min or velocities (μmoles/min) if the original, undiluted specific activity of the radioactive substrate is used to calculate v_i

Equation III-17a can be solved for the concentration of unlabeled substrate present:

$$\boxed{[S] = (K_m + [S^*])\frac{1-a}{a}} \qquad \text{(III-17b)}$$

(See also Chapter Two, Section O. The concentration of unlabeled S can also be calculated from $K_{m_{app}}$ as given by equation II-84, provided the true K_m is known).

Competitive Product Inhibition Where [S]+[P] is Constant (Regulation Via "Energy Charge")

Consider a system in which the substrate and product are interconvertible, but the total pool of [S]+[P] remains essentially constant. The rate of the S→P reaction will depend on the relative concentrations of the substrate and the product which competes with the substrate for the enzyme. Because [S]+[P] is constant, an increase in [S] automatically means that [P] must decrease. Consequently, any increase in [S] is accompanied by a decrease in the degree of competitive product inhibition. The velocity curve can be concave (decreasing slope), convex (increasing slope), or linear, depending on the relative affinities of the enzyme for S and P. To simplify matters, we will assume that K_{eq} is very large (because V_{max_r} is very small) so that even at the lowest [S]/[P] ratio the observed initial velocity is the true forward velocity of the S→P reaction, uncomplicated by the P→S reaction. This assumption eliminates the $[P]/K_{eq}$ term from the numerator of equation II-20. Alternately, we can assume that the reaction yields two products, one of which is removed in a subsequent reaction. Under either of these conditions the velocity equation is:

$$\frac{v}{V_{max}} = \frac{[S]}{K_S\left(1+\frac{[P]}{K_P}\right)+[S]} \qquad \text{(III-18)}$$

Figure III-9 shows the velocity curves for a system where $[S]+[P]=10K_S$. Note that it is possible to obtain very steep (convex) curves if $K_P < K_S$. Steep velocity curves are usually associated with multisite enzymes that display cooperative binding and possess specific effector sites. In the present system, the [S]/[P] ratio exerts a very sensitive control over the velocity when $K_P < K_S$, yet only a single binding site for S and P is involved. Atkinson and co-workers (1970) have shown that a number of ATP-utilizing enzymes are strongly inhibited by their product, ADP or AMP. The initial velocities of these reactions are markedly influenced by the ATP-ADP-AMP

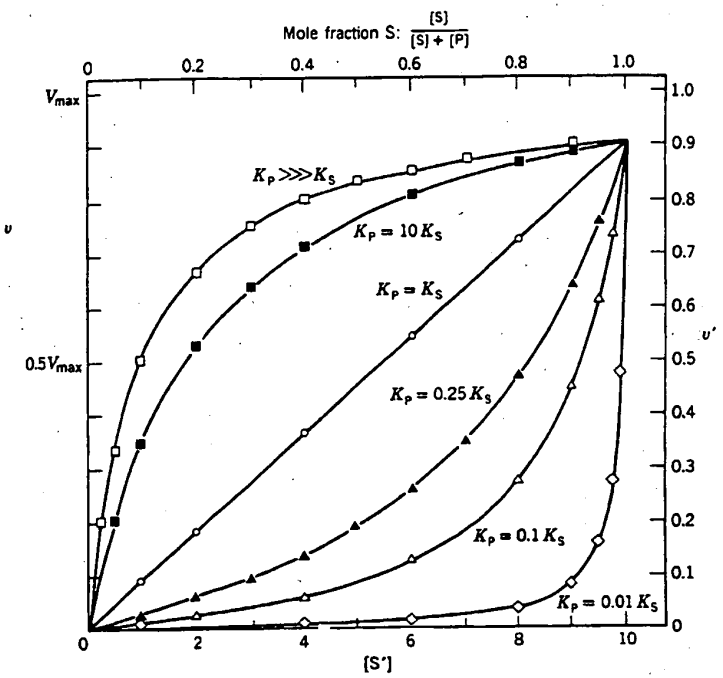


Fig. III-9. The v versus [S] plots where the total pool of [S]+[P] is constant at $10K_S$ and P has some affinity for E.

balance, called the "energy charge" of the system, where:

$$\text{"energy charge"} = \frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]} \qquad \text{(III-19)}$$

The use of "energy charge" in place of the [ATP]/[ADP] or [ATP]/[AMP] ratios represents an attempt to simulate a given total adenine nucleotide pool under conditions that would exist *in vivo* where (presumably) adenylate kinase maintains the three nucleotides at equilibrium:

$$\text{ATP} + \text{AMP} \overset{\text{adenylate kinase}}{\rightleftharpoons} 2\text{ADP}$$

Thus "energy charge" represents the mole fraction of adenine nucleotides represented by ATP or its equivalent ($\frac{1}{2}$ADP). The adenylate system is analogous to an electrochemical storage battery. The system is fully charged when all adenylate is present as ATP ("energy charge" = 1.0), and completely discharged when all adenylate is present as AMP ("energy charge" = 0). A system in which all the ATP had been converted to ADP would have an "energy charge" of 0.5. After equilibration via adenylate kinase, the "energy charge" would still be 0.5, since the total concentration of phosphate anhydride bonds would be unchanged. (Energy charge can also be defined as one half the phosphate anhydride bonds per adenosine.) The distribution of adenylates as a function of the "energy charge" is shown in Figure III-10. Many biosynthetic (i.e., energy utilizing) reactions are promoted by a high energy charge and inhibited by a low energy charge while the converse is true for energy-producing reactions. For example, Figure III-11 shows the velocity response to the [S]/[P] ratio for two enzymes, one of which catalyzes an essentially irreversible ATP-utilizing reaction (indicated as S→P) while the other catalyzes an essentially irreversible ATP-generating reaction (indicated as P→S). In both cases, $K_P = 0.1K_S$. The pool of [S] + [P] is fixed at $10K_S$. The velocities are given by the usual equations taking into account the product inhibition by P in the S→P reaction and the product inhibition by S in the P→S reaction:

$$\frac{v}{V_{max}} = \frac{[S]}{K_S\left(1 + \frac{[P]}{K_P}\right) + [S]} \qquad (\text{for } S \longrightarrow P)$$

$$\frac{v}{V_{max}} = \frac{[P]}{K_P\left(1 + \frac{[S]}{K_S}\right) + [P]} \qquad (\text{for } P \longrightarrow S)$$

If, *in vivo*, the mole fraction of [S] is poised at about 0.9 (for the particular parameters chosen), then the velocities of the S-utilizing and S-generating reactions will proceed at about $0.5V_{max}$ and small changes in the [S]/[P] ratio will tend to reestablish the original [S]/[P] ratio. (When [S] decreases, the velocity of the S→P reaction slows up while that of the P→S reaction increases.) If we consider only the S→P reaction, we see that the "energy charge" model can provide an effective "off-on" switch; that is, for a wide range of S concentrations, the velocity of the reaction can be relatively low and insensitive to increasing [S]. Then, for a relatively small increase in [S], v can increase markedly. To be an effective control system, K_P must be significantly less than K_S and the total concentration of [S] + [P] must be

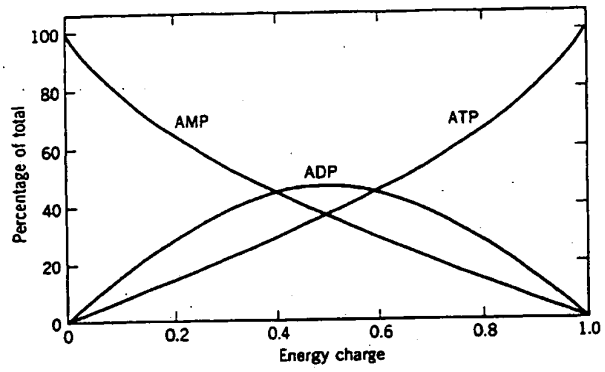


Fig. III-10. Variation of energy charge with distribution of AMP, ADP, and ATP.

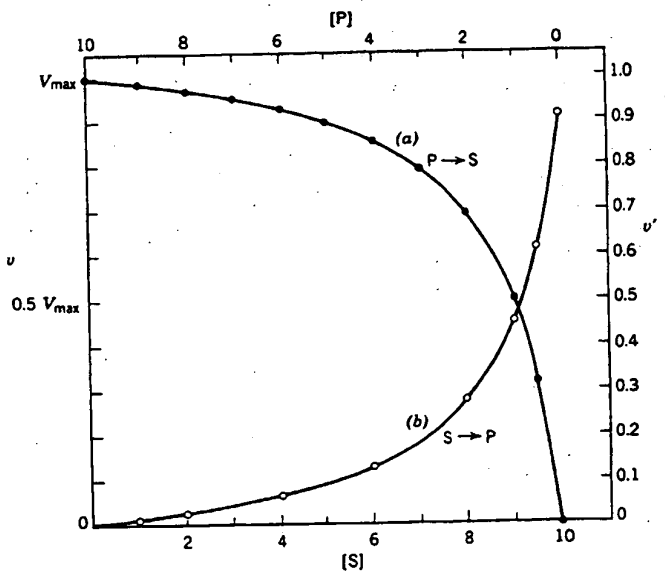


Fig. III-11. Velocity response to changing [S]/[P] ratio where [S]+[P] is constant. (*a*) A P-utilizing enzyme. (*b*) An S-utilizing enzyme. The product of one enzyme is the substrate of the other and vice versa; $K_P = 0.1$, $K_S = 1.0$, [S]+[P]=10.

large compared to K_S. (If $[S]+[P]=100K_S$, the velocity curves would be essentially the same as those shown in Figure III-11 for the same K_S and K_P values.) In this respect, it is noteworthy that the intracellular levels of adenine nucleotides are quite high compared to their K_m values. It seems likely that many biological oxidation-reduction reactions will be regulated by an analogous "reduction charge," that is, the [NADH]/[NADH+NAD$^+$] or [NADPH]/[NADPH+NADP$^+$] ratio. For most dehydrogenases involved in energy metabolism, K_{NADH} is less than K_{NAD^+}, which is exactly the condition necessary for a steep velocity response to the "reduction charge." Furthermore, the intracellular concentration of pyridine nucleotides is high compared to their K_m values (another required condition). The pyruvic dehydrogenase of *E. coli* responds to both the adenylate "energy charge" and the oxidation level of the NADH+NAD$^+$ pool. The response of pyruvic dehydrogenase to the adenylate "energy charge" can be treated as described in activation system A-5 (Chapter Five) where the enzyme activity is regulated by the [I]/[A] ratio and neither effector is a substrate or product of the reaction. Other factors, in addition to the [S]+[P] concentration and the relative K_S and K_P values, can influence the velocity response to "energy charge" or "reduction charge." These factors include (*a*) the concentrations of effectors which alter K_S or K_P, (*b*) the concentration of Mg^{2+} (when the true S and P species binding to the enzyme are the Mg complexes), and, for reactions involving two or more substrates and/or products, (*c*) the kinetic mechanism, and (*d*) the number and nature of dead-end complexes that can form. Regulation via "energy charge" may have been one of the first control devices evolved by living cells. In its simplest form, the model requires only effective competition between P and S for a single binding site. Examples of enzymes regulated by the energy charge are (*a*) citrate cleavage enzyme from rat liver, which produces extramitochondrial acetyl-*S*-CoA for fatty acid biosynthesis via the reaction:

$$\text{ATP} + \text{citrate} + \text{CoASH} \rightleftharpoons \text{acetyl-}S\text{-CoA} + \text{oxalacetate} + \text{ADP} + P_i$$

and (*b*) phosphoribosylpyrophosphate (PRPP) synthetase of *E. coli*, which furnishes PRPP for histidine, trytophan, and purine and pyrimidine nucleotides via the reaction:

$$\text{ATP} + \text{ribose-5-phosphate} \rightleftharpoons \text{PRPP} + \text{AMP}$$

It is noteworthy that ADP is much better than AMP as an inhibitor of PRPP synthetase. Thus ADP must have a higher affinity than AMP for the AMP-ATP site, or ADP binds to a distinct regulatory site. In either case, the "energy charge" of the system controls the velocity of the reaction, since a